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Creatine and Pregnancy Outcomes- A Prospective Cohort Study in Low Risk Pregnant Women: Study Protocol

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TITLE
Creatine and Pregnancy Outcomes- A Prospective Cohort Study in Low Risk Pregnant Women: Study Protocol

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ABSTRACT

Introduction: The creatine kinase circuit is central to the regulation of high-energy phosphate metabolism and the maintenance of cellular energy turnover. This circuit is fuelled by creatine, an amino acid derivative that can be obtained from a diet containing animal products, and by synthesis in the body *de novo*. A recent retrospective study conducted in a cohort of 287 pregnant women determined that maternal excreted levels of creatine may be associated with fetal growth. This prospective study design aims to overcome some of the limitations associated with the previous study and thoroughly characterise creatine homeostasis throughout gestation in a low risk pregnant population.

Methods and analysis: This study is recruiting women with a singleton low risk pregnancy who are attending Monash Health, in Melbourne, Australia. Maternal blood and urine samples, along with dietary surveys, are collected at 5 time-points during pregnancy and at delivery. Cord blood and placenta (including membranes and cord) are collected at birth. A biobank of tissue samples for future research is being established. Primary outcome measures will include creatine, creatine kinase and associated metabolites in antenatal bloods and urine, cord bloods and placenta; along with molecular analysis of the creatine transporter (SLC6A8) and synthesising enzymes arginine: glycine aminotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT) in placental tissues. Secondary outcome measures will assess dietary protein intake over pregnancy and any associations with maternal creatine, pregnancy and birth outcomes.

Ethics and dissemination: Ethical approval was granted in August 2015 from Monash Health (Ref: 14140B) and Monash University (Ref: 7785). Study outcomes will be disseminated at international conferences and published in peer-reviewed scientific journals.

Trial Registration: ACTRN12618001558213

54 **ARTICLE SUMMARY**

- 55 • This observational study will provide comprehensive information about maternal
56 adaptations to creatine homeostasis during pregnancy, with each participant providing
57 repeated biological samples across gestation and at birth (total of 6 time-points per
58 participant).
- 59 • The recruiting sites will provide a study population with diverse ethnic, socio-
60 economic and dietary backgrounds, to ensure our findings are broadly applicable.
- 61 • The establishment of a bio- and data-bank (<2,000 individual biological samples) will
62 facilitate further research in the low-risk pregnancy setting.
- 63 • As this is a study of low risk pregnant women, it is unlikely to be powered to identify
64 associations between maternal creatine levels and poor pregnancy outcomes. Results
65 will be primarily descriptive.

67 **KEYWORDS**

68 creatine kinase circuit, placenta, nutrition, diet, fetal growth restriction, fetal hypoxia

70 **WORD COUNT** 2,538

79 INTRODUCTION

80 Cells with high energy turnover utilise the creatine kinase circuit to buffer fluctuations in
81 ATP supply and demand. [1]. Creatine is critical for this pathway, and can be obtained from a
82 diet containing fish, meat or dairy, as well as being produced by the body endogenously, via a
83 two-step enzymatic reaction (*de novo* synthesis) [2-4]. Creatine synthesis involves the
84 enzyme, arginine: glycine aminotransferase (AGAT) converting the amino acids arginine and
85 glycine to the creatine precursor guanidinoacetate (GAA). Methionine then donates a methyl
86 group to GAA to produce creatine, in a secondary reaction catalysed by guanidinoacetate
87 methyltransferase (GAMT). Creatine is taken up by cells via the specific creatine transporter
88 SLC6A8 [5].

89 Dietary creatine supplementation has been studied extensively in non-pregnant humans,
90 primarily as an ergogenic aid to elite athlete training, due to its enhanced ability to supply
91 energy to cells with high energy demand such as skeletal muscle, smooth muscle and brain
92 tissue [6-10]. Despite the high energy demands of pregnancy [11, 12] and the extensive
93 research contributing to our understanding of pregnancy induced hormonal effects on many
94 amino acids and protein availability, little is known about the role of creatine in supporting
95 energy homeostasis in the mother and developing baby [12-15].

96 Studies conducted in preclinical animal models provide evidence to suggest that creatine is a
97 critical cellular energy metabolite for pregnancy and that maternal dietary creatine
98 supplementation during gestation reduces perinatal mortality and severe multi-organ
99 morbidity after hypoxic insults [14, 16-20]. Our previous retrospective collaborative study in
100 a pregnant human cohort showed maternal creatine levels appear to be related to fetal growth
101 and that increased creatine in the mother's urine is associated with increased birth weight
102 centile and length of her baby [21]. Heazell et al., also demonstrated in a matched case-
103 control study that creatine levels were reduced by 20% in serum from women who had an

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3 104 adverse pregnancy outcome (composite of stillbirth; preterm birth; small for gestational age;
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5 105 or perinatal asphyxia), after reporting reduced fetal movements, compared to those who had a
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7 106 healthy outcome [22]. These data support the theory that there is a creatine requirement
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9 107 during pregnancy. Most recently, a seminal study describing the expression of the creatine
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11 108 synthesising enzymes AGAT and GAMT, and the production of creatine by human placental
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13 109 tissue *in vitro*, suggests that the placenta may contribute to meeting maternal and fetal
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15 110 creatine requirements during pregnancy [23]. Taken together, preclinical and observational
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17 111 clinical studies indicate that creatine may be an essential metabolite during pregnancy and
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19 112 that adequate levels of creatine during pregnancy may be critical for optimal fetal growth and
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21 113 survival.

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24 114 The prospective study outlined in this protocol will characterise creatine homeostasis in a low
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26 115 risk pregnant population across gestation and at birth. The overall aim of this study is to
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28 116 further our understanding of the creatine kinase circuit in pregnancy. Specific considerations
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30 117 will include whether dietary preferences impact maternal creatine concentrations, the role of
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32 118 the placenta in creatine production, and whether maternal creatine concentrations are
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34 119 associated with pregnancy outcomes.

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40 121 *Objectives*

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42 122 1. Determine maternal concentrations of creatine, creatine kinase, arginine, glycine and
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44 123 methionine in blood and urine samples over 5 time points throughout pregnancy and
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46 124 at birth.
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48 125 2. Determine placental and cord blood concentrations of creatine, creatine kinase,
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50 126 arginine, glycine and methionine, along with molecular analysis of the creatine
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52 127 content, synthesis and transport in placental tissues at birth.
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3 128 3. Determine if maternal dietary intake of protein affects creatine concentrations across
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5 129 pregnancy.
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7 130 4. Determine whether there is any association between creatine concentrations across
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9 131 pregnancy and at birth with subsequent pregnancy and neonatal outcomes,
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11 132 specifically, fetal birth weight and length.
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134 **METHODS AND ANALYSIS**

135 *Study design*

136 A prospective observational cohort study in pregnant women, developed in reference to the
137 STROBE guidelines for cohort studies [24] and the Global Pregnancy CoLaboration site
138 (CoLab) guidelines [25].

139 *Setting*

140 Pregnant women attending low risk antenatal clinics and planning to birth at Monash Health,
141 Melbourne, Victoria, are screened for suitability. Monash Health is one of the largest
142 obstetric centres servicing Melbourne, Australia, and registers over 8000 births a year across
143 3 sites. All sites provide for low risk models of care.

144 *Participants/Recruitment*

145 Women aged 18-40 years, who have a singleton low risk pregnancy are invited to participate.
146 Women who have a known significant pre-existing major medical condition or who have
147 been assessed as high risk or have a multiple pregnancy are excluded (Table 1). As
148 pregnancy is a dynamic state, women can develop conditions or subsequent diagnoses' as
149 pregnancy progresses. Women who have a significant change in their health status or the

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status of their pregnancy, who require transfer of care to a high-risk clinic, are subsequently excluded (Table 2).

Women are approached by the researcher and the study aims and requirements discussed in detail. If women express an interest, a patient information and consent form (PICF) is provided. Women either choose to consent at the first, or subsequent visit. Consenting women may choose to biobank their samples for future perinatal research studies approved by Monash Health.

After providing informed consent, women complete a 24-hour food recall dietary questionnaire recording their previous days' food intake and the last five-hour intake prior to blood and urine sample collection. Identical samples and surveys are then collected at 4 subsequent antenatal visits every 3-6 weeks thereafter, and at birth. (Figure 1). At the first and the last research visit, women complete an online food frequency survey, *Dietary Questionnaire for Epidemiological Studies* (DQES, V2). Women receive a birth kit at the 24-28-week antenatal visit and are reminded to bring this to the hospital on day of delivery. The kit contains collection apparatus and detailed instructions for staff on sample collection and storage.

Primary outcome measures

Concentrations of maternal blood and urine creatine, creatine kinase, arginine, glycine and methionine at 5 time points during gestation, and cord vein and artery plasma and placental at birth. Measures of placental mRNA and protein expression of creatine transporter (SLC6A8), AGAT, GAMT and creatine kinases and placental enzymatic activity of AGAT and GAMT, determining capacity for placental creatine storage and synthesis [23].

173 *Secondary outcome measures*

174 Macro and micro nutrient dietary intake of women will be analysed in Foodworks 8 (Xyris
175 software) to determine if variations in dietary intake are associated with creatine
176 concentration.

177 Frequency and portion sizes of major food groups (before and during pregnancy) will be
178 measured using the food frequency survey, DQES V2. Frequency and portion sizes of major
179 food groups will be determined from the raw data and analysed by the Cancer Council of
180 Victoria's purpose made software program. A report on each participant will be provided. For
181 each participant, a scale will be attributed based on the major food groups and their portion
182 sizes. Responses will be converted to daily equivalent frequencies (DEF)[26]. The DEF and
183 portion sizes (multiplied by the portion size factor) will be used to calculate average daily
184 intake of the foods listed in the FFQ, this is then combined with data from NUTTAB95 to
185 calculate nutrient intakes [27].

186 Socio-demographic, pregnancy and birth outcomes data are also collected. Socio-
187 demographic parameters include maternal age, country of birth, ethnicity, and education
188 level. Pregnancy parameters include BMI at booking and gestational weight gain, significant
189 antenatal events, such as diagnosis of Gestational Diabetes Mellitus (GDM),
190 hospitalisations', enhanced fetal monitoring due to suspected fetal growth restriction, type of
191 onset of labour, labour stage time points, mode of delivery, blood loss, drug use during labour
192 and colour of liquor. Neonatal parameters include gestation at birth, gender, apgar scores,
193 weight, height and head circumference and length of hospital stay.

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195 *Sample collection and processing*

196 Antenatal Sample Collection: Blood is collected into lithium heparin tubes for collection of
197 plasma and kept on ice until processing (within 8 hours). Whole blood (4 x 250ul aliquots) is

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198 taken before subsequent centrifugation for isolation of plasma (400g, 20 mins, 4°C). Plasma
199 is snap frozen (10 x 250µl aliquots) and stored at -80°C. Maternal urine is kept on ice, then
200 centrifuged before freezing (10 x 500µl aliquots) and stored at -80°C. Date and times are
201 recorded for sample collection, sample processing, start and completion, and subsequent
202 freezer storage.

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204 Blood and Urine Processing: Amino acids and metabolites will be measured using Triple-
205 Quadrupole Mass Spectrometer coupled to Liquid Chromatography (LC-QqQ-MS), to
206 determine the concentrations of creatine, GAA, Phosphocreatine (PCr), arginine, glycine and
207 methionine in maternal blood and urine throughout pregnancy and in cord blood at birth [28-
208 31]. Creatine kinase will be measured in maternal blood and urine throughout pregnancy and
209 in cord blood and placenta at birth using a commercially available creatine kinase activity
210 assay.

211 Placental Processing: The placenta is trimmed of membranes and cord (1cm long cord
212 segment placed in OCT and frozen, membrane rolled and fixed in buffered formalin) before
213 obtaining placental weight. For molecular and biochemical analyses, 4 x ~2cm² pieces of
214 placenta from 4 healthy cotyledons (1 in each quadrant of the placenta) are sampled. These
215 full thickness pieces are washed in 4 sequential saline washes to remove excess blood. One
216 square is dissected into two pieces, 1 fixed in buffered formalin and the other placed in OCT
217 and frozen. Remaining squares are dissected into 0.2cm² pieces, pooled (8 x 5 piece
218 aliquots), and stored at -80°C for future mRNA and protein assessment of the creatine
219 transporter (SLC6A8); the creatine synthesising enzymes (AGAT and GAMT) and creatine
220 kinases (mitochondrial and cytosolic).

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224 *Potential Sources of Bias*

225 Selection bias and loss to follow up: We are recording the total number of women who are
226 approached and are potentially eligible for the study. Numbers of participants subsequently
227 excluded or withdrawn are recorded. Potential selection or sample bias, along with loss to
228 follow up will be reported in subsequent publications. Loss to follow up is minimized with
229 timing of research sampling coinciding with standard clinical care. Women routinely receive
230 a reminder message prior to their next appointment.

231 Unpredictable nature of birth: To enhance birth sample collection, women are provided with
232 a birth kit and reminded at subsequent appointments to bring this on presentation to hospital.
233 A computerized alert is placed in their electronic health record. A study sticker is attached to
234 the hand held maternity record to alert staff to study participation. Monash Health midwives
235 are involved in the birth sample collection. Feedback and reporting of study milestones and
236 achievements occurs routinely to enhance staff commitment and engagement.

237 Maternal diet determination: Whilst the DQES and 24-hour food recall surveys are both
238 validated tools to determine macro and micronutrient intake, all currently available diet
239 assessment tools are prone to bias and are not well validated in pregnant populations [32-34].
240 To minimise recall bias within the 24-hour food recall surveys, these are conducted over 5
241 time points and cross referenced with the researcher at each time point to enhance participant
242 recall. Multi pass food interview techniques are also employed to enhance recall and validity
243 of data assessment and enhance correct classification of macronutrients in pregnancy.

244 Misclassification of maternal factors/confounder: Gestational weight gain in pregnancy is
245 often poorly captured during routine antenatal visits. We record women's weight at each
246 research time point over pregnancy, on the same industrial scales in the antenatal clinic.
247 Whilst pre-pregnancy weight is self-reported, we determine first BMI at the earliest visit

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248 using digital scales and height measures. Country of birth may not always reflect ethnicity so

249 to minimise this bias we establish both country of birth and ethnicity.

250 Sample blinding: Samples are de-identified at the time of collection and given a sequential

251 identification number. Scientists analysing the tissue samples are blinded to the maternal

252 demographics and pregnancy and birth outcomes.

253 Data Handling: De-identified data is collected, entered and stored in our custom secure

254 database by the study coordinator. Sample processing forms are entered via a Google Drive

255 secure network and linked to de-identified data via a unique identifier.

256

257 *Sample-size and statistical analysis*

258 This study will be the first prospective study of creatine and associated metabolites across

259 pregnancy and at birth in a normal healthy pregnant population. It overcomes the limitations,

260 in regards to generalizability and bias in the diet measurements in our previously published

261 study. Findings from this study will inform future studies of effect sizes and associations.

262 Objectives 1 and 2 are descriptive only. In regard to Objectives 3 and 4, to our knowledge

263 there is no software that allows sample size determination for multi-level mixed models

264 regression. As such, no formal power calculations have been undertaken. Despite the

265 limitations of our previous study, we were sufficiently powered to determine associations

266 between plasma and urine creatine and birth weight. We have therefore determined a sample

267 size of 300 for this prospective cohort study.

268 All data will be assessed for normality. Appropriate descriptive statistics of the study sample

269 will be tabulated. The association between maternal age group (<20 years, 20-30 years and

270 30 plus), BMI (<19, 19-24.9, 25-29.9 and >=30), maternal ethnicity, diet, GWG, and urine

271 and plasma creatine over pregnancy will be determined using linear mixed models. Maternal

272 concentrations of creatine, creatine kinase, arginine, glycine and methionine in blood and

urine samples will be summarized and graphically presented over the 5 time points. The correlation between circulating and excreted creatine, amino acids and metabolites, at each of the gestation points, will also be determined.

As this is the first prospective human work on the creatine kinase circuit at birth in both cord blood and the placenta, we will also determine the correlation and agreement (ICC) between placental and cord creatine concentrations at birth. Placental and cord blood concentrations of creatine, creatine kinase, arginine, glycine and methionine, along with molecular analysis of the creatine transporter (SLC6A8) and synthesising enzymes arginine: glycine aminotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT) in placental tissues will be graphically determined at birth. The association between potential confounders such as dietary intake, body mass index (BMI), physical activity level (PAL), and gestational weight gain, (GWG) across each time point in pregnancy and maternal creatine (plasma and urine) over pregnancy will be assessed. Multivariate linear mixed models will be used to determine the associations between creatine concentrations (and associated factors), maternal diet over pregnancy as well as with growth outcomes adjusting for potential confounders.

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289 **ETHICS AND DESSEMINATION**

This study protocol was approved, as described above (with subsequent minor amendments), in August 2015 by Monash Health Human Research Ethics approval number 14140B and Monash University approval number 7785. The increased blood sampling and 5h abstinence from meat/fish were the primary ethical considerations for our study. These were addressed prior to ethics approval. Study outcomes will be disseminated at international conferences and published in peer-reviewed scientific journals. Lay reports will be made available to study participants upon request.

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DISCUSSION

This is a prospective cohort study, in low risk pregnant women, to measure creatine over pregnancy and at birth. This study will enhance our understanding of the impact diet has on maternal creatine homeostasis, and whether maternal *de novo* synthesis maintains creatine homeostasis across pregnancy despite variations in dietary intake. These studies will also enhance our understanding of the role the placenta plays in creatine homeostasis during pregnancy. Data collected will help establish the framework on which to build future studies of maternal dietary creatine supplementation during gestation to improve pregnancy outcomes. In addition, the development of a new biobank of antenatal samples will also provide a valuable asset for future research endeavours in this field.

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427 AUTHOR'S CONTRIBUTIONS

428 HD conceived the study design. MDT performed power and sample size calculations. HD
429 and SE developed and executed protocols for sample collection and processing. DdeG drafted
430 the manuscript and leads study recruitment and coordination.

431

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438 fellowships. No funding body had a role in study design, data collection, analysis,
439 interpretation or writing of this manuscript.

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441 COMPETING INTERESTS STATEMENT

442 The authors declare that they have no 'competing interests' in this section.

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5 469 **FIGURE LEGENDS**
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7 470 **Figure 1. Schematic Overview of Study Recruitment and Sample Collection**
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Table 1: Comparison of inclusion and exclusion criteria

Primary Inclusion Criteria	Primary Exclusion Criteria
<ul style="list-style-type: none">• Age 18-40 inclusive• Singleton pregnancy• Low risk pregnancy (based on past medical or obstetric history)• Attending Monash Health for birth• Between 10-20 weeks’ gestation at recruitment (+/- 1 week)• Good understanding/reading of English	<ul style="list-style-type: none">• Multiple pregnancy• Type 1/Type 2 Diabetes• High risk pregnancy (requiring care in a high-risk pregnancy clinic)• Model of care (not attending most appointments at tertiary centre)• Use of creatine supplements in pregnancy• Non-English speaking/requiring interpreter

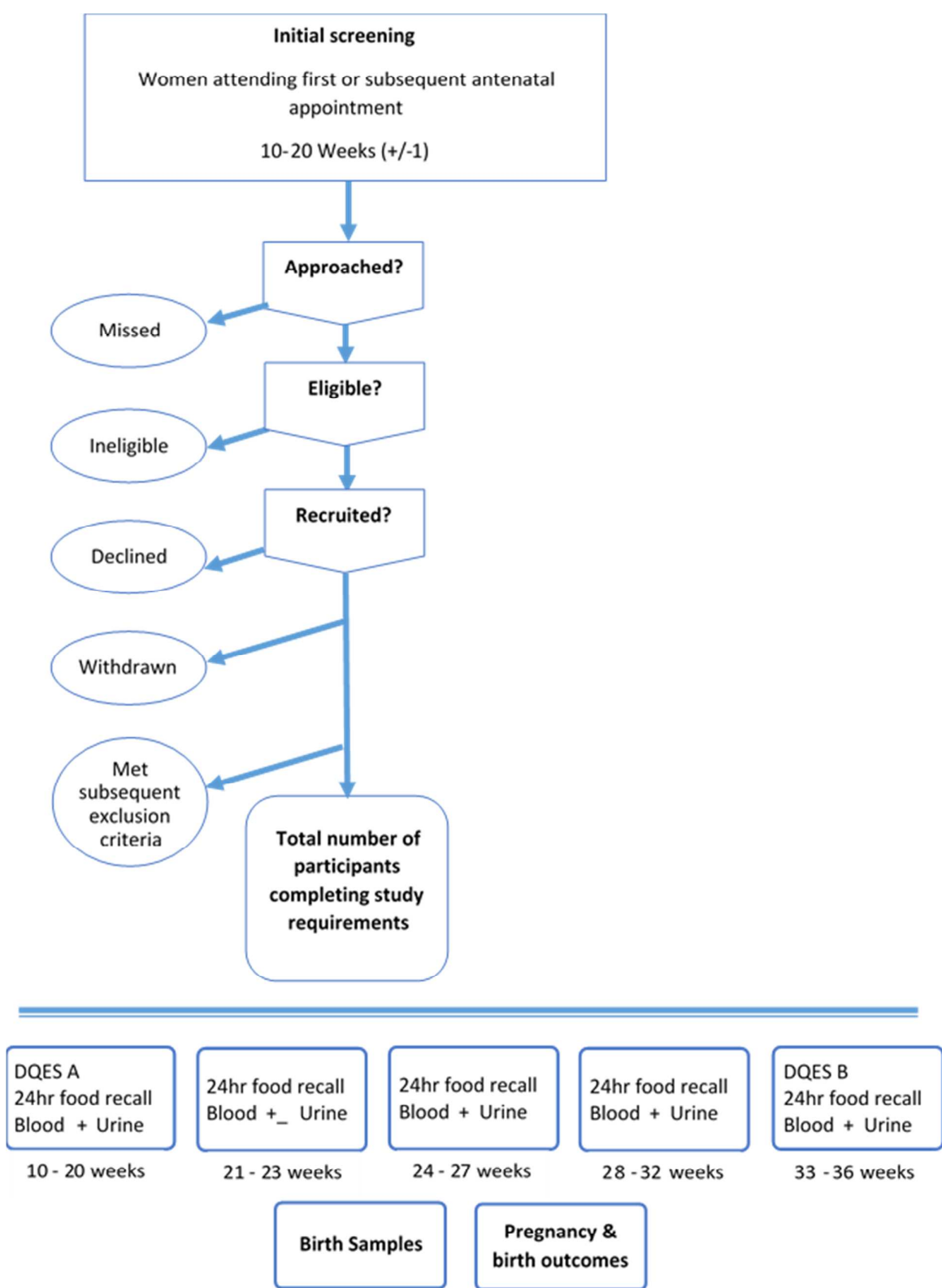
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511 **Table 2: Subsequent exclusion criteria**

• Major congenital fetal abnormality
• Change of birth venue/model of care, during pregnancy
• Disclosure of ongoing substance use/alcohol or drug dependency
• Exacerbation of previously stable medical condition now requiring active intervention and transfer to a high-risk pregnancy clinic
• Development of significant new medical/pregnancy condition requiring active intervention and transfer to high risk pregnancy clinic

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For peer review only

BMJ Open

Creatine and Pregnancy Outcomes- A Prospective Cohort Study in Low Risk Pregnant Women: Study Protocol

Journal:	<i>BMJ Open</i>
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Primary Subject Heading:	Obstetrics and gynaecology
Secondary Subject Heading:	Nutrition and metabolism
Keywords:	creatine kinase circuit, placenta, Nutrition < TROPICAL MEDICINE, Fetal growth restriction, fetal hypoxia

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TITLE

Creatine and Pregnancy Outcomes- A Prospective Cohort Study in Low Risk Pregnant Women: Study Protocol

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ABSTRACT

Introduction: The creatine kinase circuit is central to the regulation of high-energy phosphate metabolism and the maintenance of cellular energy turnover. This circuit is fuelled by creatine, an amino acid derivative that can be obtained from a diet containing animal products, and by synthesis in the body *de novo*. A recent retrospective study conducted in a cohort of 287 pregnant women determined that maternal excreted levels of creatine may be associated with fetal growth. This prospective study aims to overcome some of the limitations associated with the previous study and thoroughly characterise creatine homeostasis throughout gestation in a low risk pregnant population.

Methods and analysis: This study is recruiting women with a singleton low risk pregnancy who are attending Monash Health, in Melbourne, Australia. Maternal blood and urine samples, along with dietary surveys, are collected at 5 time-points during pregnancy and at delivery. Cord blood and placenta (including membranes and cord) are collected at birth. A biobank of tissue samples for future research is being established. Primary outcome measures will include creatine, creatine kinase and associated metabolites in antenatal bloods and urine, cord bloods and placenta; along with molecular analysis of the creatine transporter (SLC6A8) and synthesising enzymes arginine: glycine aminotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT) in placental tissues. Secondary outcome measures include dietary protein intake over pregnancy and any associations with maternal creatine, pregnancy events and birth outcomes.

Ethics and dissemination: Ethical approval was granted in August 2015 from Monash Health (Ref: 14140B) and Monash University (Ref: 7785). Study outcomes will be disseminated at international conferences and published in peer-reviewed scientific journals.

Trial Registration: ACTRN12618001558213

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3 54 **ARTICLE SUMMARY**
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6 55 • This observational study will provide comprehensive information about maternal
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8 56 adaptations to creatine homeostasis during pregnancy, with each participant providing
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10 57 repeated biological samples across gestation and at birth (total of 6 time-points per
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12 58 participant).

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15 59 • The recruiting sites will provide a study population with diverse ethnic, socio-economic
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17 60 and dietary backgrounds, to ensure our findings are broadly applicable.

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19 61 • The establishment of a bio- and data-bank (<2,000 individual biological samples) will
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21 62 facilitate further research in the low-risk pregnancy setting.

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24 63 • As this is a study of low risk pregnant women, it is unlikely to be powered to identify
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26 64 associations between maternal creatine levels and poor pregnancy outcomes. Results
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28 65 will be primarily descriptive.

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33 67 **KEYWORDS**
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35 68 creatine kinase circuit, placenta, nutrition, diet, fetal growth restriction, fetal hypoxia
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79 INTRODUCTION

80 Cells with high energy turnover utilise the creatine kinase circuit to buffer fluctuations in ATP
81 supply and demand. [1]. Creatine is critical for this pathway, and can be obtained from a diet
82 containing fish, meat or dairy, as well as being produced by the body endogenously, via a two-
83 step enzymatic reaction (*de novo* synthesis) [2-4]. Creatine synthesis involves the enzyme
84 arginine: glycine aminotransferase (AGAT) converting the amino acids arginine and glycine
85 to the creatine precursor guanidinoacetate (GAA). Methionine then donates a methyl group to
86 GAA to produce creatine, in a secondary reaction catalysed by guanidinoacetate
87 methyltransferase (GAMT). Creatine is taken up by cells via the specific creatine transporter
88 SLC6A8 [5].

89 Dietary creatine supplementation has been studied extensively in non-pregnant humans,
90 primarily as an ergogenic aid to elite athlete training, due to its enhanced ability to supply
91 energy to cells with high energy demand [6-10]. Despite the increased metabolic load of
92 pregnancy [11, 12], and pregnancy induced hormonal effects on many amino acids and protein
93 availability, little is known about the role of creatine in supporting energy homeostasis in the
94 mother and developing baby [12-15].

95 Studies conducted in preclinical animal models provide evidence to suggest that creatine is a
96 critical cellular energy metabolite for pregnancy and that maternal dietary creatine
97 supplementation during gestation reduces perinatal mortality and severe multi-organ morbidity
98 after hypoxic insults [14, 16-20]. Our previous retrospective collaborative study in a pregnant
99 human cohort showed maternal creatine levels appear to be related to fetal growth, with
100 increased creatine concentrations in the mother's urine being associated with increased birth
101 weight centile and length of her baby [21]. Heazell et al., also demonstrated in a matched case-
102 control study that creatine levels were reduced by 20% in serum from women who had an
103 adverse pregnancy outcome (composite of stillbirth; preterm birth; small for gestational age;

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3 104 or perinatal asphyxia) [22]. These data support the theory that there is a creatine requirement
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5 105 during pregnancy. Most recently, a seminal study describing the expression of the creatine
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8 106 synthesising enzymes AGAT and GAMT, and the production of creatine by human placental
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10 107 tissue *in vitro*, suggests that the placenta may contribute to meeting maternal and fetal creatine
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12 108 requirements during pregnancy [23]. Taken together, preclinical and observational clinical
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14 109 studies indicate that creatine may be an essential metabolite during pregnancy and that
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17 110 adequate levels of creatine during pregnancy may be critical for optimal fetal growth and
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19 111 survival.
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21 112 The prospective study outlined in this protocol will characterise creatine homeostasis in a low
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23 113 risk pregnant population across gestation and at birth. The overall aim of this study is to further
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25 114 our understanding of the creatine kinase circuit in pregnancy. Specific considerations will
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27 115 include whether dietary preferences impact maternal creatine concentrations, the role of the
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29 116 placenta in creatine production, and whether maternal creatine concentrations are associated
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32 117 with pregnancy outcomes.
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39 119 *Objectives*

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41 120 1. Determine maternal concentrations of creatine, creatine kinase, arginine, glycine and
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43 121 methionine in blood and urine samples over 5 time points throughout pregnancy and at
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45 122 birth.
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47 123 2. Determine placental and cord blood concentrations of creatine, creatine kinase,
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49 124 arginine, glycine and methionine, along with molecular analysis of the creatine content,
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51 125 synthesis and transport in placental tissues at birth.
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53 126 3. Determine if maternal dietary intake of protein affects creatine concentrations across
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55 127 pregnancy.
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4. Determine whether there is any association between creatine concentrations across pregnancy and at birth with maternal characteristics in pregnancy and neonatal outcomes, specifically, fetal birth weight and length.

METHODS AND ANALYSIS

Study design

A prospective observational cohort study in pregnant women, developed in reference to the STROBE guidelines for cohort studies [24] and the Global Pregnancy CoLaboration site (CoLab) guidelines [25].

Patient and Public Involvement

Participants were not asked or offered the opportunity to participate in the study design. The researchers did consider the study requirements in relation to pregnancy care and scheduled all appointments to coincide women's visits to antenatal clinics.

Setting

Pregnant women attending low risk antenatal clinics and planning to birth at Monash Health, Melbourne, Victoria.

Participants/Recruitment

Women aged 18-40 years, who have a singleton low risk pregnancy are invited to participate. Women who have a known significant pre-existing major medical condition or who have been assessed as high risk are excluded (Table 1). As pregnancy is a dynamic state, women can develop conditions or subsequent diagnoses' as pregnancy progresses. Women who have a

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3 150 significant change in their health status or the status of their pregnancy, or who require transfer
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5 151 of care to a high-risk clinic, are subsequently excluded (Table 2).
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10 153 Women are approached by the researcher and the study aims and requirements discussed in
11
12 154 detail. If women express an interest, a patient information and consent form (PICF) is provided.
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14 155 Women either choose to consent at the first, or subsequent visit to the antenatal clinic (between
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16 156 10-20 weeks). After providing informed consent, blood and urine samples and 24-hour food
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18 157 recalls are collected at 5 antenatal visits between 10-20 weeks (time of consent), 21-23 weeks,
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20 158 24-27 weeks, 28-32 and 33-36 weeks, and at birth (Figure 1). At the first and the last antenatal
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22 159 visit, women complete an online food frequency survey, *Dietary Questionnaire for*
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24 160 *Epidemiological Studies* (DQES, V2). Women receive a birth kit at the 24-28-week antenatal
25
26 161 visit and are reminded to bring this to the hospital on day of delivery. The kit contains collection
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28 162 apparatus and detailed instructions for staff on sample collection and storage. Consenting
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30 163 women may choose to biobank their samples for future perinatal research studies approved by
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32 164 Monash Health.
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41 166 *Primary outcome measures*
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43 167 Concentrations of creatine, creatine kinase, arginine, glycine and methionine are measured in
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45 168 maternal plasma and urine at 5 time points during gestation, in cord vein and arterial plasma,
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47 169 and placental tissue at birth. Placental mRNA and protein expression of the creatine transporter
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49 170 (SLC6A8), AGAT, GAMT and creatine kinases will also be analysed, along with placental
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51 171 enzymatic activity of AGAT and GAMT, to determine placental creatine synthesis and storage
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173 *Secondary outcome measures*

174 Macro and micro nutrient dietary intake of women will be analysed in Foodworks 8 (Xyris
175 software) to determine if variations in dietary intake are associated with creatine concentration.
176 Frequency and portion sizes of major food groups (before and during pregnancy) will be
177 measured using the food frequency survey, DQES V2. Frequency and portion sizes of major
178 food groups will be determined from the raw data and analysed by the Cancer Council of
179 Victoria's purpose made software program. A report on each participant will be provided. For
180 each participant, a scale will be attributed based on the major food groups and their portion
181 sizes. Responses will be converted to daily equivalent frequencies (DEF) [26]. The DEF and
182 portion sizes (multiplied by the portion size factor) will be used to calculate average daily
183 intake of the foods listed in the FFQ, this is then combined with data from NUTTAB95 to
184 calculate nutrient intakes [27].

185 Socio-demographic data, pregnancy events and birth outcomes data are also collected. Socio-
186 demographic parameters include maternal age, country of birth, ethnicity, and education level.
187 Relevant medical history will capture any pre-existing clinical variables such as
188 hypothyroidism or other correctable nutritional deficiencies. Pregnancy parameters include
189 body mass index (BMI) at booking, blood pressure readings, and gestational weight gain over
190 pregnancy. Significant antenatal events, include diagnosis of Gestational Diabetes Mellitus
191 (GDM), hospitalisations', enhanced maternal monitoring due to blood pressure changes, or
192 enhanced fetal monitoring due to suspected fetal growth restriction. Labour and delivery
193 outcomes will be captured and will include, type of onset of labour, labour stage time points,
194 drug use during labour and colour of liquor, mode of delivery and blood loss. Neonatal
195 parameters include gestation at birth, gender, apgar scores, weight, height and head
196 circumference and length of hospital stay.

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Sample collection and processing

Antenatal sample collection: Blood is collected into lithium heparin tubes for collection of plasma and kept on ice until processing (note: creatine is stable in whole blood, kept on ice, for up to 8 hours). Whole blood (4 x 250ul aliquots) is taken before subsequent centrifugation for isolation of plasma (400g, 20 mins, 4°C). Plasma aliquots (10 x 250µl) are then stored at -80°C. Urine is collected and kept on ice until processing (within 8 hours). The sample is transferred to a 50 mL falcon tube and centrifuged (400g, 20 mins, 4°C), before being aliquoted (10 x 500µl) and stored at -80°C. Date and times are recorded for sample collection, sample processing start and completion, and subsequent freezer storage.

Placental processing: The placenta is trimmed of membranes and cord (1cm long cord segment placed in OCT and frozen, membrane rolled and fixed in buffered formalin) before obtaining placental weight. For molecular and biochemical analyses, 4 x ~2cm² pieces of placenta from 4 healthy cotyledons (1 in each quadrant of the placenta) are sampled. These full thickness pieces are washed in 4 sequential saline washes to remove excess blood. One square is dissected into two pieces, 1 fixed in buffered formalin and the other placed in OCT and frozen. Remaining squares are dissected into 0.2cm² pieces, pooled (8 x 5 piece aliquots), and stored at -80°C for future molecular analysis.

Sample Analysis

Amino acids and metabolites will be measured using Triple-Quadrupole Mass Spectrometer coupled to Liquid Chromatography (LC-QqQ-MS), to determine the concentrations of creatine, GAA, Phosphocreatine (PCr), arginine, glycine and methionine in maternal blood and urine throughout pregnancy and in cord blood at birth [28-31]. Creatine kinase will be measured in maternal blood and urine throughout pregnancy and in cord blood and placenta at

birth using a commercially available creatine kinase activity assay. RNA and protein will be extracted from placental tissue using standard laboratory techniques. RT-qPCR and western blot analysis will be used to assess expression patterns of the creatine transporter (SLC6A8); the creatine synthesising enzymes (AGAT and GAMT) and creatine kinases (mitochondrial and cytosolic).

Potential Sources of Bias

Selection bias and loss to follow up: We are recording the total number of women who are approached and are potentially eligible for the study. Numbers of participants subsequently excluded or withdrawn are recorded. Potential selection or sample bias, along with loss to follow up will be reported in subsequent publications. Loss to follow up is minimized with timing of research sampling coinciding with standard clinical care. Women routinely receive a reminder message prior to their next appointment.

Unpredictable nature of birth: To enhance birth sample collection, women are provided with a birth kit and reminded at subsequent appointments to bring this on presentation to hospital. A computerized alert is placed in their electronic health record. A study sticker is attached to the hand held maternity record to alert staff to study participation. Monash Health midwives are involved in the birth sample collection. Feedback and reporting of study milestones and achievements occurs routinely to enhance staff commitment and engagement.

Maternal diet determination: Whilst the DQES and 24-hour food recall surveys are both validated tools to determine macro and micronutrient intake, all currently available diet assessment tools are prone to bias and are not well validated in pregnant populations [32-34]. To minimise recall bias within the 24-hour food recall surveys, these are conducted over 5 time points and cross referenced with the researcher at each time point to enhance participant recall.

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3 247 Multi pass food interview techniques are also employed to enhance recall and validity of data
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5 248 assessment and enhance correct classification of macronutrients in pregnancy.
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7 249 Misclassification of maternal factors/confounder: Gestational weight gain in pregnancy is often
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9 250 poorly captured during routine antenatal visits. We record women’s weight at each research
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11 251 time point over pregnancy, on the same industrial scales in the antenatal clinic. Whilst pre-
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13 252 pregnancy weight is self-reported, we determine first BMI at the earliest visit using digital
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15 253 scales and height measures. Country of birth may not always reflect ethnicity so to minimise
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17 254 this bias we establish both country of birth and ethnicity.
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19 255 Sample blinding: Samples are de-identified at the time of collection and given a sequential
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21 256 identification number. Scientists analysing the tissue samples are blinded to the maternal
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23 257 demographics and pregnancy and birth outcomes.
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25 258 Data Handling: De-identified data is collected, entered and stored in our custom secure
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27 259 database by the study coordinator. Sample processing forms are entered via a Google Drive
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29 260 secure network and linked to de-identified data via a unique identifier.
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33 262 *Sample-size and statistical analysis*
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38 263 This study will be the first prospective study of creatine and associated metabolites across
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40 264 pregnancy and at birth in a normal healthy pregnant population. It overcomes the limitations,
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42 265 in regards to generalizability and bias in the diet measurements in our previously published
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44 266 study. Findings from this study will inform future studies of effect sizes and associations.
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46 267 Objectives 1 and 2 are descriptive only. In regard to Objectives 3 and 4, to our knowledge there
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48 268 is no software that allows sample size determination for multi-level mixed models regression.
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50 269 As such, no formal power calculations have been undertaken. Despite the limitations of our
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52 270 previous study, we were sufficiently powered to determine associations between plasma and
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271 urine creatine and birth weight. We have therefore determined a sample size of 300 for this
272 prospective cohort study.

273 All data will be assessed for normality. Appropriate descriptive statistics of the study sample
274 will be tabulated. The association between maternal age group (<20 years, 20-30 years and 30
275 plus), BMI (<19, 19-24.9, 25-29.9 and ≥30), maternal ethnicity, diet, GWG, and urine and
276 plasma creatine over pregnancy will be determined using linear mixed models. Maternal
277 concentrations of creatine, creatine kinase, arginine, glycine and methionine in blood and urine
278 samples will be summarized and graphically presented over the 5 time points. The correlation
279 between circulating and excreted creatine, amino acids and metabolites, at each of the gestation
280 points, will also be determined.

281 As this is the first prospective human work on the creatine kinase circuit at birth in both cord
282 blood and the placenta, we will also determine the correlation and agreement (ICC) between
283 placental and cord creatine concentrations at birth. Placental and cord blood concentrations of
284 creatine, creatine kinase, arginine, glycine and methionine, along with molecular analysis of
285 the creatine transporter (SLC6A8) and synthesising enzymes arginine: glycine
286 aminotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT) in placental
287 tissues will be graphically determined at birth. The association between potential confounders
288 such as dietary intake, blood pressure, body mass index (BMI), physical activity level (PAL),
289 and gestational weight gain, (GWG) across each time point in pregnancy and maternal creatine
290 (plasma and urine) over pregnancy will be assessed. Multivariate linear mixed models will be
291 used to determine the associations between creatine concentrations (and associated factors),
292 maternal diet over pregnancy as well as with growth outcomes adjusting for potential
293 confounders.

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ETHICS AND DESSEMINATION

This study protocol was approved, as described above (with subsequent minor amendments), in August 2015 by Monash Health Human Research Ethics approval number 14140B and Monash University approval number 7785. The increased blood sampling and 5h abstinence from meat/fish were the primary ethical considerations for our study. These were addressed prior to ethics approval. Study outcomes will be disseminated at international conferences and published in peer-reviewed scientific journals. Lay reports will be made available to study participants upon request.

DISCUSSION

This is a prospective cohort study, in low risk pregnant women, to measure creatine homeostasis over pregnancy and at birth. This study will enhance our understanding of the potential impact maternal factors, including diet and ethnicity, may have on maternal creatine homeostasis. These studies will also enhance our understanding of the role the placenta plays in creatine homeostasis during pregnancy. It is beyond the scope of this study to capture all pregnancy populations. As this is a study of low risk pregnant women, it is unlikely to be powered to identify associations between maternal creatine levels and poor pregnancy outcomes. Results will be primarily descriptive; however, data collected in this population may be used to compare to higher risk pregnancy populations in the future. Overall, this research will help establish the framework on which to build future studies of maternal dietary creatine supplementation during gestation to improve pregnancy outcomes. In addition, the development of a new biobank of antenatal samples will also provide a valuable asset for future research endeavours in this field.

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AUTHOR'S CONTRIBUTIONS

HD conceived the study design. MDT performed power and sample size calculations. HD and SE developed and executed protocols for sample collection and processing. DdeG drafted the manuscript and leads study recruitment and coordination.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no 'competing interests' in this section.

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FIGURE LEGENDS

Figure 1. Schematic Overview of Study Recruitment and Sampling Regime. Pregnancy events and characteristics include socio-demographic parameters, relevant medical history, body mass index (BMI), blood pressure and gestational weight. Birth outcomes include labour and delivery outcomes, type of onset of labour, labour stage time points, drug use, colour of liquor, mode of delivery, blood loss, and neonatal parameters including gestational age, gender, apgar scores, weight, height, head circumference and length of hospital stay. DQES – Dietary Questionnaire for Epidemiological Studies. Weeks – number of weeks' gestation.

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Table 1: Comparison of inclusion and exclusion criteria

Primary Inclusion Criteria	Primary Exclusion Criteria
<ul style="list-style-type: none">• Age 18-40 inclusive• Singleton pregnancy• Low risk pregnancy (based on past medical or obstetric history)• Attending Monash Health for birth• Between 10-20 weeks’ gestation at recruitment (+/- 1 week)• Good understanding/reading of English	<ul style="list-style-type: none">• Multiple pregnancy• Type 1/Type 2 Diabetes• High risk pregnancy (requiring care in a high-risk pregnancy clinic)• Model of care (not attending most appointments at tertiary centre)• Use of creatine supplements in pregnancy• Non-English speaking/requiring interpreter

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519 **Table 2: Subsequent exclusion criteria**

• Major congenital fetal abnormality
• Change of birth venue/model of care, during pregnancy
• Disclosure of ongoing substance use/alcohol or drug dependency
• Exacerbation of previously stable medical condition now requiring active intervention and transfer to a high-risk pregnancy clinic
• Development of significant new medical/pregnancy condition requiring active intervention and transfer to high risk pregnancy clinic

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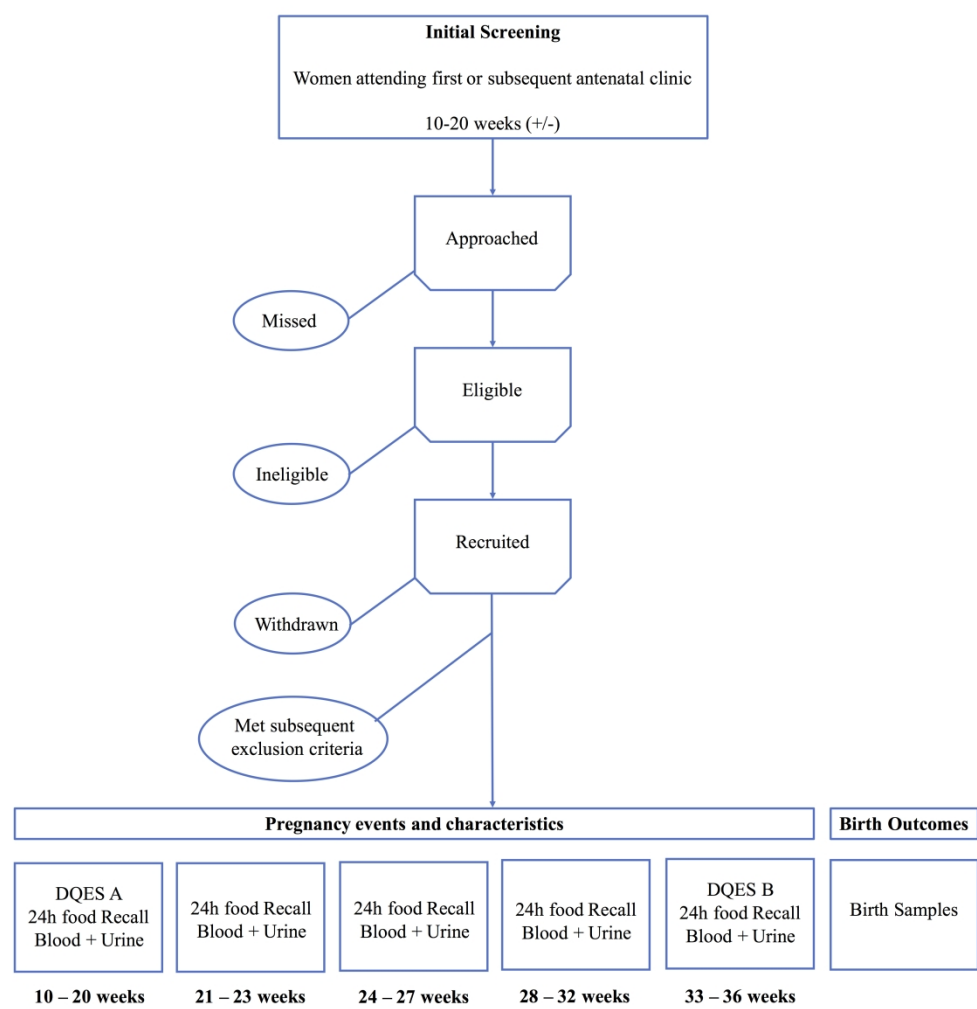


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